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## DICHLOROMETHANE UTILIZATION IN A PACKED-BED REACTOR IN THE PRESENCE OF VARIOUS ELECTRON ACCEPTORS

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**Abstract**—Dichloromethane, added as sole source of carbon and energy, was utilized by microorganisms in a packed-bed reactor under carbon dioxide-, sulfate-, nitrate- and nitrite-reducing conditions. Only in the presence of nitrite (4 mM) was the transformation of dichloromethane partly inhibited. The maximum transformation rate for dichloromethane under carbon dioxide reducing conditions was  $1.25 \text{ kg m}^{-3} \text{ d}^{-1}$ . Carbon dioxide, acetate and formate were detected as (intermediate) products of dichloromethane transformation in the reactor, indicating that it was a fermentative process. Both acetate and formate, when formed, were further utilized. The type of microorganisms that utilized formate and acetate depended on the electron acceptor present in the reactor. When carbon dioxide was the only electron acceptor available, acetate and formate were utilized by methanogens as indicated by methane production. When sulfate, nitrate or nitrite were present in the reactor, acetate and formate were utilized by sulfate-, nitrate- or nitrite-reducing microorganisms, respectively. Inhibition of methanogens with 2-bromoethane sulfonic acid or of sulfate reduction with molybdate had no effect on the utilization of dichloromethane in enrichment cultures from the reactor. Also the presence of nitrate or nitrite was not necessary for the transformation of dichloromethane. These results suggested that neither methanogens nor sulfate-, nitrate- and nitrite-reducers were involved in the transformation of dichloromethane but that these organisms only utilized acetate and formate, the products of dichloromethane fermentation in the reactor. © 1999 Elsevier Science Ltd. All rights reserved

**Key words**—dichloromethane, fermentation, redox conditions, packed-bed reactor

### INTRODUCTION

Dichloromethane (DCM) and chloromethane are the only chlorinated hydrocarbons of which it is known that they can serve as growth substrates for aerobic (Brunner *et al.*, 1980; Lapat-Polasko *et al.*, 1984; Kohler-Staub *et al.*, 1986; Bader and Leisinger, 1994) as well as anaerobic (Freedman and Gossett, 1991; Mägli *et al.*, 1996) microorganisms. Both aerobic (Rittmann and McCarty, 1980; Gälli and Leisinger, 1985; Stucki, 1990) and anaerobic (Stromeyer *et al.*, 1991; Winkelbauer and Kohler, 1991) microbial transformation of DCM have been exploited in bioreactors for wastewater and groundwater treatment. Since aerobic transformation is a much faster process than its anaero-

bic counterpart (Leisinger *et al.*, 1994), it is favored for practical applications. Anaerobic treatment would be an attractive option when besides DCM other chlorinated hydrocarbons that cannot be treated under aerobic conditions, like carbon tetrachloride and tetrachloroethene, are present. Moreover, anaerobic processes have the advantage that little biomass is produced, oxygen is unnecessary, and that less energy is needed for operation of the reactor (Stromeyer *et al.*, 1991).

Little is known about the effect of different primary electron acceptors—such as sulfate and nitrate—on the anaerobic transformation of DCM. This information is important in view of the application of anaerobic biotransformation of DCM for *in situ* and on-site biological groundwater remediation at contaminated sites under different or even multiple electron acceptor conditions. In this study we examined the transformation of DCM in an anaerobic packed-bed reactor under anaerobic conditions. The maximum transformation capacity of

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the reactor and the mass balance of DCM transformation under these conditions are presented. Furthermore the effect of primary electron acceptors on the anaerobic transformation of DCM were examined by adding different concentrations of sulfate, nitrate and nitrite to the reactor influent.

## MATERIAL AND METHODS

### Packed-bed reactor studies

The experiments were performed in an anaerobic upflow packed-bed reactor (glass, height 32 cm, inside diameter 4.42 cm, volume 492 ml) (de Best *et al.*, 1997) packed with polyurethane foam (PUR) particles (80% v/v) ( $5 \times 5 \times 6$  mm, Bayer BV, Mijdrecht, The Netherlands) mixed with digested sludge (20% v/v) from the Kralingseveer wastewater treatment plant (Rotterdam, The Netherlands). The packed-bed reactor was wrapped with aluminum foil to prevent growth of phototrophs. The packed-bed reactor contained 5 sample ports at different heights along the reactor (0 cm = influent; 8, 16, 24, 32 = effluent).

The packed-bed reactor was continuously fed with an anaerobic non-sterile mineral medium containing (mg/l)  $\text{K}_2\text{HPO}_4$  (8),  $\text{KH}_2\text{PO}_4$  (3.6),  $\text{NaHCO}_3$  (40),  $\text{NH}_4\text{Cl}$  (26.6),  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (101.6),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (62.6), resazurine (1). From a trace element solution, 0.125 ml/l were added. The trace element solution contained (mg/l)  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (2800),  $\text{H}_3\text{BO}_3$  (50),  $\text{Al}_2(\text{SO}_4)_3 \cdot 16\text{H}_2\text{O}$  (118.3),  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  (50),  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (92.8), EDTA (500),  $\text{ZnCl}_2$  (50),  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{27} \cdot \text{H}_2\text{O}$  (50),  $\text{CoCl}_2$  (27.3),  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$  (91.6), 1 ml HCl (37%). The medium was continuously purged with a mixture of  $\text{N}_2$  and  $\text{CO}_2$  (99.5%/0.5%) (Hoek Loos BV, Dieren, The Netherlands) to remove all oxygen.

The medium ( $\text{pH } 7.3 \pm 0.2$ ) was pumped into the packed-bed reactor by means of a peristaltic pump with marprene tubing (Watson Marlow, England). All other tubing was either viton or Teflon. Dichloromethane, acetate and  $\text{Na}_2\text{S}$  (42  $\mu\text{M}$ , to maintain reducing conditions) were added to the medium as a concentrated solution at the influent of the packed-bed reactor with a syringe pump. The hydraulic retention time in the packed-bed was 24 h. All experiments were carried out at 25°C.

### Batch culture studies

Batch culture studies were started to obtain an enrichment culture from the reactor. Four different media were tested for their ability to support DCM transformation. Besides the medium that was also used for the reactor (medium 1) and the effluent of the reactor (medium 2), two other media were tested. *Medium 3*: (in g/l of demineralized water)  $\text{KH}_2\text{PO}_4$  (0.43),  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  (0.53),  $\text{NH}_4\text{Cl}$  (0.3),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (0.12),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.13), resazurine (0.0005). The medium also contained (per liter) 1 ml of trace element solution (de Best *et al.*, 1997) and 1 ml of a vitamin solution. The vitamin solution contained (mg/l): biotin (2), folic acid (2), riboflavin (5), thiamine (5), cyanocobalamin (5), nicotinamide (5), *p*-aminobenzoic acid (5). *Medium 4*: (in g/l of demineralized water)  $(\text{NH}_4)_2\text{HPO}_4$  (0.080),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.20), resazurine (0.001) and 5 ml trace element solution (see above). The different media were purged with a mixture of  $\text{CO}_2$  and  $\text{N}_2$  (0.5%/99.5%, 700 ml/min) for 45 min. After purging,  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$  (67 mg/l) and  $\text{NaHCO}_3$  (100 mg/l) were added to media 2 and 3. The media were transferred to 120 or 250 ml bottles in an anaerobic glovebox. Bottles (120 ml) contained 60 ml of medium and were closed with teflon-lined butyl rubber stoppers and aluminum crimp seals. Bottles (250 ml) contained 180 ml of medium and were

closed with viton stoppers and an aluminum screw cap. After sterilization the batch cultures were inoculated and DCM (48  $\mu\text{M}$ ) was added from a concentrated stock solution. The cultures were incubated on a shaker (100 rpm) at 25°C in the dark and analyzed regularly for chlorinated hydrocarbons.

### Analytical methods

Dichloromethane and chloromethane were quantified by headspace gas chromatography. Liquid samples (100–1000  $\mu\text{l}$ ) were injected in 10 ml headspace autosampler vials with teflon-lined butyl rubber stoppers and aluminum crimp seals. The final volume was adjusted to 2 ml with demineralized water. The vials were analyzed using a Hewlett-Packard 19395A headspace sampler connected to gas chromatograph equipped with an ECD and a CP-Sil 5CB column (de Best *et al.*, 1997). Calibration samples were analyzed according to the same method to adjust for air/water partitioning. A four-point curve was used.

Carbon dioxide and methane concentrations were determined after separation on a CarboPlot P7 column in a gas chromatograph equipped with an FID and a methanizer (de Best *et al.*, 1997). For samples from the reactor, liquid samples (2 ml) were injected in 10 ml headspace autosampler vials with teflon-lined butyl rubber stoppers and aluminum crimp seals and equilibrated at 80°C for 45 min. A volume of 50  $\mu\text{l}$  of the headspace was injected into the GC by hand with a 100  $\mu\text{l}$  Hamilton gas- and liquid-tight syringe. For batch cultures, 50  $\mu\text{l}$  of the headspace was injected into the GC. A four point calibration curve was used for quantification.

Sulfate, nitrate, nitrite and chloride were determined after separation on an IONPAC AG9-SC guard column and IONPAC AG9-SC anion column (Dionex, Breda, The Netherlands) using an ion chromatograph equipped with a conductivity detector, thermal stabilizer and ASRS suppressor (de Best *et al.*, 1997).

Acetate and formate concentrations were determined with an enzymatic test combination (Boehringer, Mannheim, Germany).

### Chemicals

All chemicals were obtained from commercial companies. DCM and chloromethane were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Sodium acetate was obtained from Fischer Scientific ('s Hertogenbosch, The Netherlands). Sodium sulfate, potassium nitrate and sodium nitrite were purchased from Boom (Meppel, The Netherlands). Calibration gases (carbon dioxide, methane) were obtained from AGA (Hoogvliet, The Netherlands).

## RESULTS

### Transformation of dichloromethane in an anaerobic packed-bed reactor

The transformation of DCM (25  $\mu\text{M}$ ) was studied in an anaerobic packed-bed reactor, inoculated with digested sludge. Acetate (0.83 mM) served as an electron donor. After a lag period of 12 days, during which all DCM added was recovered in the effluent, at day 13 DCM was completely transformed in the reactor whilst simultaneous methanogenesis and sulfate reduction occurred (Table 1). Chloromethane or other chlorinated hydrocarbons were not found as transformation products. Acetate was completely utilized. The occurrence of methane production (741  $\mu\text{M}$ ) and sulfate reduction (54  $\mu\text{M}$ )

Table 1. Effect of acetate concentration on dichloromethane (25  $\mu\text{M}$ ) transformation in an anaerobic packed-bed reactor

Influent ( $\mu\text{M}$ )		Effluent ( $\mu\text{M}$ )			
$\text{CH}_2\text{Cl}_2$	$\text{CH}_3\text{COOH}$	$\text{CH}_2\text{Cl}_2$ transformed	$\text{CH}_3\text{COOH}$ transformed	$\text{SO}_4^{2-}$ reduced	$\text{CH}_4$ formed
22.3	834	22.3	806	54	741
27.8 <sup>a</sup>	0	27.3	0	45	23

<sup>a</sup>Steady state, 8 weeks after omission of acetic acid from the packed-bed reactor.

indicated that both methanogenic and sulfate-reducing bacteria were responsible for acetate removal.

Omitting acetate from the influent of the reactor resulted in a slow decrease of methane production and sulfate reduction since acetate served as growth substrate for both methanogenic bacteria and sulfate-reducing bacteria. After three weeks still some methane production (23  $\mu\text{M}$ ) and sulfate reduction (45  $\mu\text{M}$ ) occurred. This could be due to conversion of organic material still present in the reactor and originating from the inoculum (digested sludge). The removal of acetate from the influent of the reactor had no effect on the transformation of DCM (Table 1). This indicated that DCM served as a growth substrate for microorganisms, as described before for anaerobic bacteria (Freedman and Gossett, 1991; Mägli *et al.*, 1996).

#### Transformation capacity and mass balance

To determine the volumetric transformation capacity of the reactor, the concentration of DCM in the influent was increased stepwise, starting at 390  $\mu\text{M}$ , until DCM was no longer completely transformed and appeared in the effluent of the reactor. Besides DCM no other substrate was added to the reactor. Carbon dioxide was the only potential electron acceptor present.

Up to a concentration of 2.1 mM, DCM was completely transformed in the reactor (Table 2). Only at a concentration of 19 mM, DCM was detected in the effluent of the reactor. At this concentration, a volumetric DCM transformation capacity of 0.31  $\text{kg m}^{-3} \text{d}^{-1}$  could be calculated for the reactor. Determination of DCM at different sample ports along the height of the reactor (see Material and Methods) indicated that DCM trans-

formation only occurred in the first 25% of the volume of the reactor. For this part of the reactor, a transformation capacity of 1.25  $\text{kg m}^{-3} \text{d}^{-1}$  was calculated.

At DCM concentrations higher than 390  $\mu\text{M}$ , a chlorine and carbon balance could be determined (Table 2). Between 91 and 102% of the chlorine appeared as chloride, indicating that DCM was completely dechlorinated. Carbon dioxide, methane and acetic acid were detected as products of DCM transformation. At a DCM concentration of 19 mM, formic acid was also found as a transformation product. The closed mass balance for carbon at the four lower DCM concentrations tested indicated that no other products were formed. At the highest DCM concentration, however, the recovery of carbon was poor because part of the gas produced did not dissolve but left the reactor as small gas bubbles. The volume of these gas bubbles and their composition were not measured and thus not included in the data presented in Table 2.

At DCM concentrations up to 2.1 mM, part of acetate formed in the first half of the reactor was utilized again at the upper half of the reactor (data not shown). Acetate was probably utilized by methanogens, as indicated by the production of methane. The presence of methanogens was confirmed by fluorescence microscopy (Doddema and Vogels, 1978).

Reaction (1) shows the overall transformation of DCM to methane, resulting in a [DCM transformed/ $\text{CH}_4$  formed]-ratio of 2. From the data presented in Table 2, an average [DCM transformed/ $\text{CH}_4$  formed]-ratio of 4.1 was calculated for the reactor. This ratio indicates that DCM was not completely transformed to methane. Part of DCM

Table 2. Effect of dichloromethane concentration on utilization of dichloromethane in an anaerobic packed-bed reactor

Influent ( $\mu\text{M}$ ) $\text{CH}_2\text{Cl}_2$	Effluent ( $\mu\text{M}$ )						Recovery (%)	
	$\text{CH}_2\text{Cl}_2$ transformed	$\text{Cl}^-$ formed	$\text{CH}_3\text{COOH}$ formed	$\text{HCOOH}$ formed	$\text{CH}_4$ formed	$\text{CO}_2$ formed	C	$\text{Cl}^-$
390	390	—	< 4	< 4	80	—	—	—
573	573	1110	14	< 4	130	474	110	97
1151	1151	2319	90	< 4	234	787	104	102
2115	2096	3980	141	< 4	446	1455	104	95
19,000	3756	6833	370	82	< 5	1482	61	91

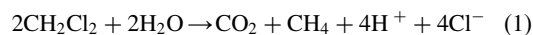
— Not determined.

Table 3. Effect of sulfate, nitrate and nitrite on the transformation of dichloromethane in an anaerobic packed-bed reactor

Electron acceptor added	Influent (μM)		Effluent (μM)							Recovery (%)	
	CH <sub>2</sub> Cl <sub>2</sub>	CH <sub>2</sub> Cl <sub>2</sub> transformed <sup>a</sup>	Cl <sup>-</sup> formed	CH <sub>3</sub> COOH formed	SO <sub>4</sub> <sup>2-</sup> reduced <sup>a</sup>	NO <sub>3</sub> <sup>-</sup> reduced <sup>a</sup>	NO <sub>2</sub> <sup>-</sup> reduced <sup>a</sup>	CH <sub>4</sub> formed	CO <sub>2</sub> formed	C	Cl <sup>-</sup>
None	0	573	1110	14	< 2	< 2	< 5	130	474	110	97
SO <sub>4</sub> <sup>2-</sup>	763	531	986	< 4	153	< 2	< 5	< 5	— <sup>b</sup>	—	93
	4109	581	1196	< 4	138	< 2	< 5	< 5	561	97	103
NO <sub>3</sub> <sup>-</sup>	3050	560	1222	< 4	< 2	660	< 5	< 5	436	78	109
NO <sub>2</sub> <sup>-</sup>	998	617	1144	< 4	< 2	< 2	989	< 5	—	—	95
	3953	611	670	< 4	< 2	< 2	1284	< 5	—	—	93

<sup>a</sup>Based on the concentration in the influent minus the concentration in the effluent.<sup>b</sup>Not determined.<sup>c</sup>A steady state was not yet reached.

was probably transformed to carbon dioxide by other bacteria, as indicated by the production of carbon dioxide (Table 2)

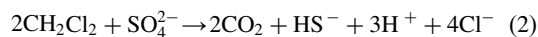


At the highest DCM concentration tested (19 mM) both acetate and formic acid, when formed, were not transformed by methanogens or other bacteria. This was probably due to an inhibitory effect of DCM and a decrease of the pH of the medium from 7.3 to 6.1, which resulted from HCl formation. As a result, acetate and formic acid accumulated.

#### Effect of sulfate on dichloromethane transformation

Sulfate is a potential electron acceptor that is often found in groundwater at DCM contaminated sites. To determine the effect of sulfate on DCM transformation, sulfate was added to the influent of the reactor. Two different sulfate concentrations were examined (Table 3).

In the absence of sulfate, DCM (573  $\mu\text{M}$ ) was completely dechlorinated. Carbon dioxide and methane were found as main transformation products but part of DCM was also converted to acetate. Sulfate reduction did not occur. Addition of sulfate had no effect on the transformation of DCM. At both sulfate concentrations tested (0.76 and 4.1 mM), DCM was completely dechlorinated according to the chlorine mass balance (Table 3). Methane production was completely inhibited when sulfate was present in the influent of the reactor. This inhibition of methanogenesis had no effect on the transformation of DCM, indicating that methanogens were not involved in this transformation. Instead of methane production, an average of 146  $\mu\text{M}$  of sulfate was reduced at both sulfate concentrations tested. Reaction (2) shows that transformation of 555  $\mu\text{M}$  of DCM (average) provides enough electrons for the reduction of 278  $\mu\text{M}$  of sulfate. The production of carbon dioxide (Table 3) indicated that part of DCM was oxidized to carbon dioxide by other microorganisms. Acetate was only found as a product of DCM transformation at several sample ports at different heights of the reactor (data not shown). No acetate was detected in the effluent of the reactor indicating that acetate, when formed, was utilized in the reactor.



When sulfate was removed from the influent, and sulfate reduction thus no longer could occur, methane production was detected again in the reactor after 5 days. DCM transformation still was complete.

#### Effect of nitrate on dichloromethane transformation

Nitrate is another primary electron acceptor

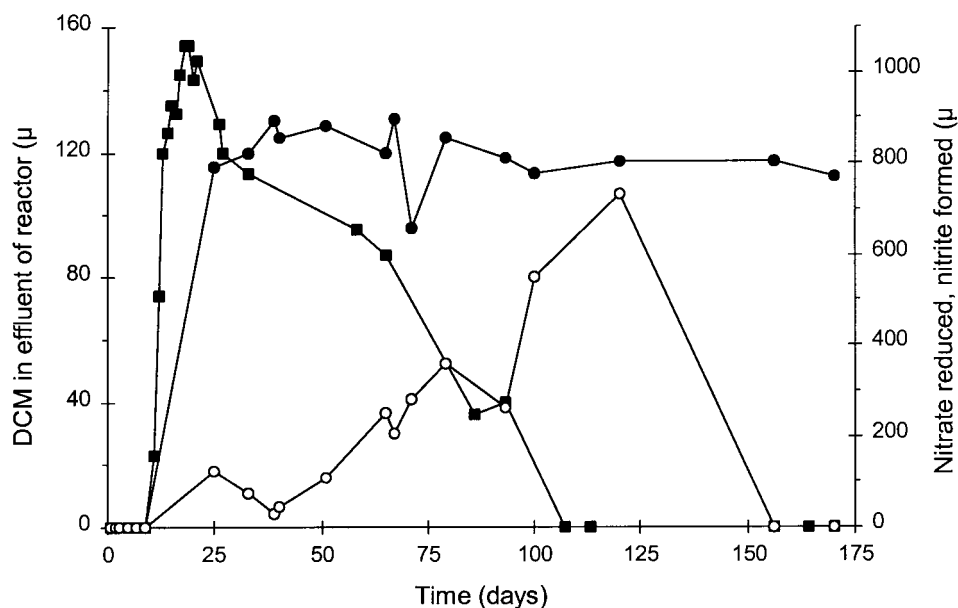


Fig. 1. Effect of nitrate (3 mM) on the transformation of dichloromethane (570  $\mu\text{M}$ ) in a packed-bed reactor. Most of the dichloromethane is removed; the remaining concentration in the effluent of the reactor is plotted. Symbols: DCM in the effluent of the reactor (■); nitrate reduced (●); nitrite formed (○).

which frequently occurs in groundwater. The effect of nitrate (3 mM) on the transformation of DCM in the reactor was examined (Fig. 1). Before nitrate was added (day 1–10), DCM (570  $\mu\text{M}$ ) was completely dechlorinated according to the chlorine mass balance (Table 3). Carbon dioxide (474  $\mu\text{M}$ ) and methane (130  $\mu\text{M}$ ) were found as products of DCM transformation. Part of DCM was converted to acetate (14  $\mu\text{M}$ ).

The transformation of DCM was only partially

and temporarily inhibited by the presence of nitrate. When an excess of nitrate (3 mM) was added to the influent of the reactor at day 10, the concentration of DCM detected in the effluent of the reactor rapidly increased until it stabilized at about 155  $\mu\text{M}$  after 7 days. Next, DCM transformation recovered and 100 days after nitrate was first added to the reactor, DCM was again completely dechlorinated.

When nitrate was present in the influent of the reactor, methane production by methanogens was

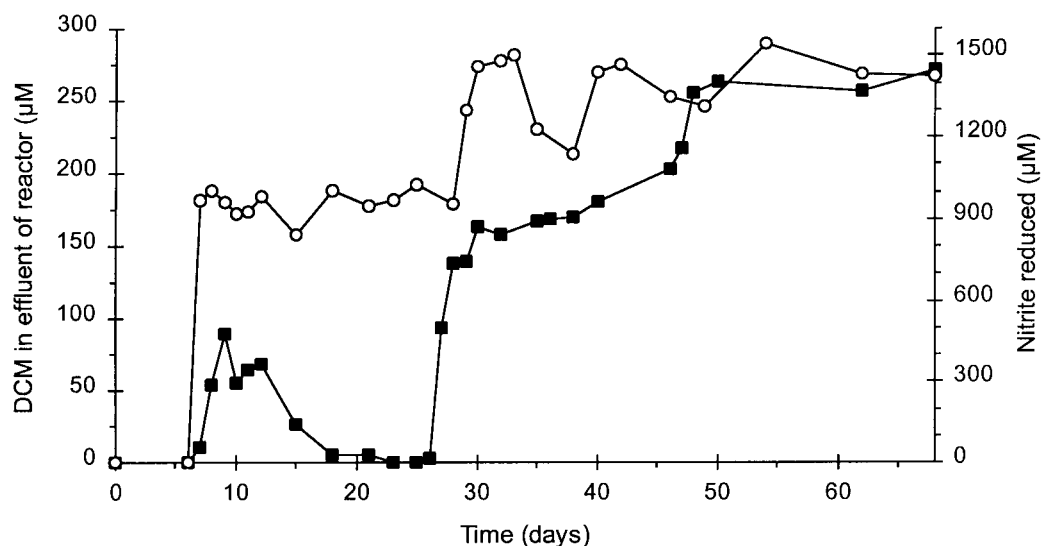
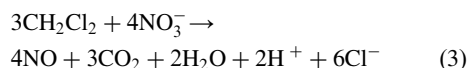


Fig. 2. Effect of nitrite on the transformation of dichloromethane ( $\pm 600 \mu\text{M}$ ) in a packed-bed reactor. Symbols: DCM in the effluent of the reactor (■); nitrite reduced (○).

completely inhibited. Instead of a methanogenic population, a nitrate reducing bacterial population developed in the reactor within 14 days after nitrate was first added. At first part of nitrate was reduced to nitrite but when a steady state was reached after 150 days, nitrite was no longer detected in the effluent of the reactor while 660  $\mu\text{M}$  of nitrate was reduced (Table 3). Nitrate can either be reduced to  $\text{NO}$ ,  $\text{N}_2\text{O}$ ,  $\text{N}_2$  or  $\text{NH}_4^+$  (Stouthamer, 1988). The electron balance of DCM oxidation to  $\text{CO}_2$  and nitrate reduction to  $\text{NO}$ ,  $\text{N}_2\text{O}$ ,  $\text{N}_2$  or  $\text{NH}_4^+$  showed that transformation of 560  $\mu\text{M}$  of DCM (Table 3) does not provide enough electrons for the reduction of all the nitrate to  $\text{NH}_4^+$ ,  $\text{N}_2$  or  $\text{N}_2\text{O}$ . This indicated that nitrate may be reduced to  $\text{NO}$  according to (overall) reaction (3). For the reduction of 660  $\mu\text{M}$  of  $\text{NO}_3^-$  to  $\text{NO}$ , oxidation of 495  $\mu\text{M}$  DCM to  $\text{CO}_2$  would be needed.



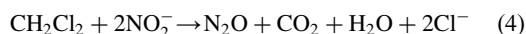
Acetate was only found as a product of DCM transformation at several sample ports at different heights of the reactor (data not shown). No acetate was detected in the effluent of the reactor indicating that acetate, when formed, was utilized in the reactor.

Two weeks after nitrate was omitted from the influent of the reactor, methane (131  $\mu\text{M}$ ) and carbon dioxide (450  $\mu\text{M}$ ) again were found as products of DCM transformation. DCM was still completely dechlorinated.

#### *Effect of nitrite on dichloromethane transformation*

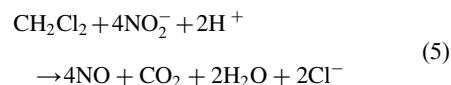
The temporary inhibition of DCM transformation by nitrate could be a result of the formation of nitrite. Nitrite is often found to be toxic for microorganisms (Tiedje, 1988). To verify this hypothesis, nitrite was added to the influent of the reactor at two different concentrations (Fig. 2).

Before addition of nitrite, DCM (550  $\mu\text{M}$ ) was completely dechlorinated under methanogenic conditions as indicated by the production of methane (131  $\mu\text{M}$ ). Addition of nitrite (1 mM) at day 6 had little effect on the transformation of DCM. The concentration of DCM in the effluent of the reactor increased to a concentration of 90  $\mu\text{M}$ , but within 12 days DCM again was completely dechlorinated according to the formation of chloride (Table 3). Methane production no longer occurred, but 989  $\mu\text{M}$  of nitrite was reduced. The electron balance indicated that nitrite was reduced to  $\text{N}_2\text{O}$  according to reaction (4). For this reduction, oxidation of 495  $\mu\text{M}$  DCM would be needed. About 605  $\mu\text{M}$  of DCM was oxidized in the reactor.



At day 26, the concentration of nitrite was

increased to 4 mM (Fig. 2). Nitrite reduction increased to an average of 1284  $\mu\text{M}$  (607  $\mu\text{mol l}^{-1} \text{d}^{-1}$ ) and about 2.7 mM of nitrite appeared in the effluent of the reactor. The presence of nitrite in the influent clearly affected DCM transformation. After a fast initial inhibition the concentration of DCM in the effluent of the reactor slowly increased further until after 68 days only 359  $\mu\text{M}$  of DCM was transformed (no steady state). According to the formation of chloride (Table 3), all of the DCM which was transformed was completely dechlorinated. The electron balance indicated that  $\text{NO}_2^-$  was reduced to  $\text{NO}$  instead of  $\text{N}_2\text{O}$  according to the following overall reaction:



Before a steady state was reached, nitrite was omitted from the influent of the reactor. Within 15 days, DCM again was completely dechlorinated. Methane production (130  $\mu\text{M}$ ) indicated that methanogens had survived and were active in the reactor.

#### *Dichloromethane utilizing enrichment culture*

Anaerobic enrichments were started in 250 ml bottles using different media. The batch cultures were inoculated with 4 ml of liquid phase from the packed-bed reactor. DCM (48  $\mu\text{M}$ ) was added as single substrate.

A DCM transforming enrichment culture could only be obtained using sterilized effluent from the reactor as medium (medium 2). With medium 1, 3 and 4, no growth on DCM was observed. This indicated that a growth factor was required that was produced/present in the reactor. Specific nutritional requirements have been reported for several respiratory-dehalogenating bacteria (Shelton and Tiedje, 1984; Holliger, 1992; Mägli *et al.*, 1995).

Within 10 days DCM (48  $\mu\text{M}$ ) was completely transformed (Fig. 3). Chloromethane was not detected as a transformation product indicating that DCM was completely dechlorinated. Repeated additions of DCM were also dechlorinated without a significant lag period (Fig. 3). Growth was observed by light microscopy. A mixed population containing at least two different gram-negative microorganisms had developed, a flagellated rod and a non-moving string of rods. In control bottles without an inoculum from the reactor or a sterilized inoculum, no DCM transformation occurred and losses were limited to 5% after 100 days.

Addition of 2-bromoethane sulfonic acid (BES; 6 mM), an inhibitor of methanogenesis, or molybdate (2 mM), an inhibitor of sulfate reduction in sulfate-reducing bacteria, to a DCM degrading enrichment culture had no effect on DCM transformation. This indicated that neither methanogenic

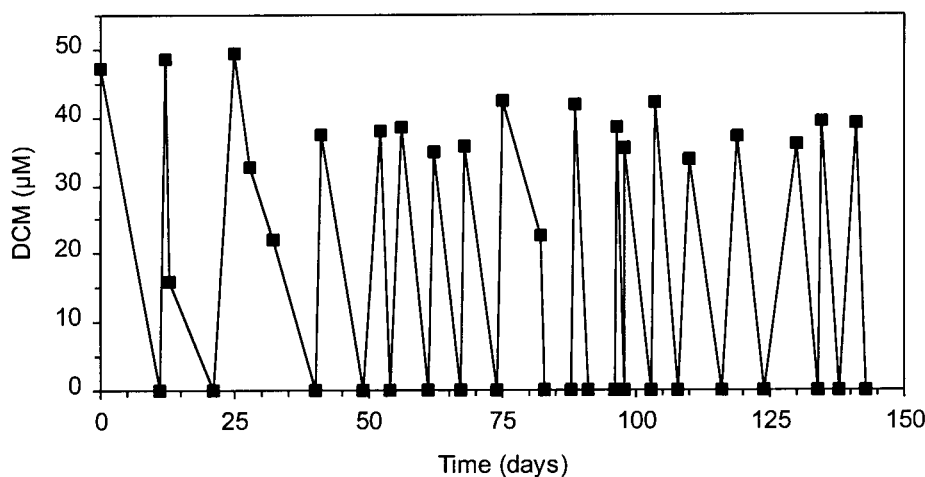


Fig. 3. Transformation of dichloromethane by an anaerobic mixed culture. DCM was added as single substrate. The sterilized effluent of a DCM degrading packed-bed reactor served as medium. Symbol: dichloromethane (■).

nor sulfate-reducing bacteria were involved in DCM transformation. Vancomycin (0.07 mM), an inhibitor of cell wall synthesis in gram positive eubacteria, completely inhibited DCM transformation. These results indicated that gram positive bacteria, like acetogens or *Clostridium* species, were involved in the transformation of DCM.

After anaerobic transfer from the enrichment cultures to fresh effluent medium, the ability to grow under anaerobic conditions on halogenated hydrocarbons other than DCM was tested. Growth was only observed on DCM and on dibromomethane (1 mM). Tetrachloromethane (1 mM), chloroform (1 mM), tetrachloroethene (1 mM), trichloroethene (1 mM), *cis*-1,2-dichloroethene (0.5 mM), *trans*-1,2-dichloroethene (0.5 mM), 1,1-dichloroethene (0.5 mM), 1,1,1-trichloroethane (1 mM), 1,1-dichloroethane (1 mM), 1,2-dichloroethane (1 mM) and chloroethane (0.5 mM) were not transformed, nor was growth observed microscopically.

#### DISCUSSION

Here we report microbial utilization of DCM in a packed-bed reactor under carbon dioxide-, sulfate-, nitrate- and nitrite-reducing conditions. Anaerobic utilization of DCM in a reactor has been described previously, but only under carbon dioxide reducing conditions (Freedman and Gossett, 1991; Stromeyer *et al.*, 1991) as a fermentative process, and under nitrate reducing conditions (Kohler-Staub *et al.*, 1995). Utilization of DCM under denitrification conditions was not a fermentative process. Instead, *Hyphomicrobium* sp. Strain DM2 coupled the reduction of nitrate to nitrite to the hydrolysis of DCM to formaldehyde, which then served as the growth substrate.

Carbon dioxide and acetate were found as (intermediate) products of DCM transformation in our reactor under all redox conditions that were examined. Under carbon dioxide reducing conditions, formate was also found as a product of DCM transformation. These products are similar to the products of DCM transformation by *Dehalobacterium formicoaceticum*, a microorganism that was isolated from a DCM utilizing fixed-bed reactor (Stromeyer *et al.*, 1991). *D. formicoaceticum* is a DCM fermenting microorganism that is able to transform DCM to acetate and formate using DCM as sole substrate for growth (Mägli *et al.*, 1996). These results suggested that DCM transformation under carbon dioxide-, sulfate-, nitrate- and nitrite-reducing conditions in the reactor was also a fermentative process. So far, fermentation of chlorinated aliphatic hydrocarbons has been limited to DCM and chloromethane (Trautnecker *et al.*, 1991; Mägli *et al.*, 1996). Both with DCM and chloromethane fermentation, acetate and formate were found as (intermediate) products of transformation.

Acetate and formate, when formed, were partly or completely utilized in the reactor. The type of microorganism that utilized acetate and formate depended on the predominating electron acceptor condition in the reactor. If carbon dioxide was the only electron acceptor available, acetate and formate were utilized by methanogens as indicated by methane production. When sulfate, nitrate or nitrite were present in the reactor, sulfate-, nitrate- and nitrite-reducing microorganisms, respectively, developed in the reactor and utilized acetate and formate. Methanogens and sulfate reducers were not involved in the transformation of DCM. Inhibition of methanogens with 2-bromoethane sulfonic acid or sulfate-reduction with molybdate had no effect



on the utilization of dichloromethane in enrichment cultures from the reactor. Nitrate- and nitrite reducers were probably also not involved in DCM transformation in the reactor since the presence of nitrate or nitrite was not necessary for DCM fermentation.

The maximum DCM elimination rate of the reactor under carbon dioxide reducing conditions, calculated for the first 25% of the volume, was  $1.25 \text{ kg DCM m}^{-3} \text{ d}^{-1}$ . This rate is much higher than the rate observed for the fermentative transformation of DCM by a mixed culture in a fixed-bed reactor under carbon dioxide reducing conditions (Stromeyer *et al.*, 1991) or the rate observed for DCM transformation by *Hyphomicrobium* sp. strain DM2 under denitrification conditions in a fed batch culture (Kohler-Staub *et al.*, 1995). However, it is still about 10 times lower than the elimination rate Stucki (1990) described for the removal of DCM in an aerobic fluidized bed reactor ( $12 \text{ kg DCM m}^{-3} \text{ d}^{-1}$ ).

Although aerobic transformation is much faster, the anaerobic process described in this paper has the advantage that DCM can be transformed with either carbon dioxide, sulfate, nitrate or nitrite as the predominant electron acceptor in the reactor. Only the presence of 4 mM of nitrite inhibited DCM transformation for a longer period of time. This inhibition could be caused by the toxicity of nitrite or NO, the expected product of nitrite reduction in the reactor, for the DCM fermenting microorganisms. Both nitrite and NO are often found to be toxic for microorganisms (Tiedje, 1988). Transformation of DCM by *Hyphomicrobium* sp. strain DM2 was also inhibited by nitrite and depended on the concentration of nitrite (Kohler-Staub *et al.*, 1995). At a nitrite concentration of 5 mM, DCM transformation was completely inhibited whereas partial inhibition of DCM transformation was observed at nitrite concentrations between 0.1 mM and 4 mM. These findings could explain why DCM transformation in our reactor was only partly inhibited in the presence of 4 mM of nitrite.

### CONCLUSIONS

Dichloromethane, added as sole source of carbon and energy, can be utilized by microorganisms in a packed-bed reactor under carbon dioxide-, sulfate-, nitrate- and nitrite-reducing conditions. Carbon dioxide, acetate and formate were detected as (intermediate) products of dichloromethane transformation in the reactor, indicating that it was a fermentative process. This was confirmed by batch experiments with an enrichment culture from the reactor. The ability to transform DCM with different electron acceptors, i.e. under different redox conditions, is important in view of practical applications of *in situ* and on-site bioremediation of contaminated

sites. Different redox conditions occur at different sites and often redox conditions may differ at one contaminated site (Chapelle, 1996; Nipshagen *et al.*, 1997). Therefore, a practical process which transforms DCM at different redox conditions can be of great importance. The maximum elimination rate of  $1.25 \text{ kg DCM m}^{-3} \text{ d}^{-1}$  detected for anaerobic DCM transformation in our reactor can also compete with transformation of DCM under aerobic conditions.

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